Sensory pathways of muscle phenotypic plasticity: Calcium signalling through CaMKII

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Summary and general discussion

In this chapter, the results of the experiments described in this thesis are summarised and suggestions for experiments are made to further elucidate the role of Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) and calcium-dependent signalling in regulating skeletal muscle adaptation.

Summary

Skeletal muscle is able to adapt its phenotype in response to increased usage, and improve its strength and fatigue resistance. Calcium-dependent signalling is presumed to increase mitochondrial biogenesis, an important adaptation to improve fatigue-resistance. CaMKII has been suggested to promote a slow-twitch, high oxidative phenotype in skeletal muscle, but its physiological role in muscle adaptation has so far not been investigated *in vivo*. The work described in this thesis aimed to elucidate the role of CaMKII in skeletal muscle *in vivo*. For this purpose, a rat model which allows for imposing defined stimulation protocols on skeletal muscle *in situ* was combined with the manipulation of CaMKII signalling by somatic gene transfer and a computational model of CaMKII signalling.

In chapter 2, the question was addressed whether CaMKII activation depends on recruitment frequency and whether its activity remains after muscle stimulation ceases. Adult rat skeletal muscle was stimulated *in situ* by different stimulation protocols after which protein levels of total CaMKII and phospho^{Thr287}-CaMKII were measured for the β_M, δ_A & δ_D/γ_B isoforms at different time points. This study consisted of two experiments. In the first experiment, *m. soleus* (SOL) and *m. gastrocnemius medialis* (GM) were stimulated with 100 electrical impulses at 10 or 150 Hz at their active slack length (i.e. a length at which no active force is produced). This protocol increased phospho^{Thr287}-CaMKII of δ_A and δ_D/γ_B isoforms in red GM, δ_A isoform in white GM, but none of the isoforms in SOL. There was no effect of frequency on the level of phospho^{Thr287}-CaMKII. A decrease in total β_m and δ_D/γ_B CaMKII was observed in GMW after 150 Hz stimulation. These results suggest that CaMKII is more strongly activated in fast-twitch muscle, and that motor neuron firing frequency is not an important factor in determining CaMKII activation.

In the second experiment, we sampled GM at rest, after determination of optimum length and at 2, 10 or 60 minutes after a two-minute isometric contraction protocol at optimum length. The following observations were made: 1) After the contractile activity required to determine the optimum length of the muscle, phospho^{Thr287}-CaMKII for the δ_A isoform was increased in red GM compared to rest. 2) Phospho^{Thr287}-CaMKII was higher in white GM compared to red GM regardless of stimulation. 3) Total CaMKII decreased over time in white GM. These results suggest that after a short contraction protocol, phospho^{Thr287}-CaMKII is transiently increased in an isoform- and muscle-dependent way. The results of the first and second experiment together suggest that CaMKII remains activated for 1 to 15 minutes after stimulation.

In chapter 3, the effects of acute CaMKII overexpression in skeletal muscle *in vivo* were investigated. α/β-CaMKII was overexpressed in adult rat *m. gastrocnemius* (GM) and *m. soleus* (SOL) by electroporation-mediated gene transfer. We hypothesised that CaMKII overexpression would increase the expression of mitochondrial genes. However, our experiments showed that CaMKII overexpression did not increase protein levels of cytochrome c oxidase IV (COXIV) in GM or SOL. The mRNA levels of oxidative phosphorylation components COXIV, COXI and transcriptional co-activator peroxisome proliferator-activated receptor gamma co-activator 1α were not different in empty- and CaMKII-transfected SOL, whereas succinate dehydrogenase subunit b mRNA was decreased in CaMKII-transfected SOL. Force parameters of transfected muscles were measured *in situ* with intact innervations and perfusion. Strength and fatigue resistance of control- and CaMKII-transfected SOL and GM did not differ. However, we observed that CaMKII overexpression caused a decrease in twitch time-to-peak force and half-relaxation time. In addition, CaMKII-overexpressing SOL fibres displayed increased sarco/endoplasmic reticulum Ca^{2+}-ATPase 2 expression compared to non-transfected fibres. Our results therefore suggest that increased CaMKII levels alone are not sufficient to increase mitochondrial biogenesis, but may be involved in calcium handling and the regulation of contractile force characteristics.

In addition to its role in mitochondrial biosynthesis, CaMKII is presumed to be involved in the regulation of a signalling pathway that controls skeletal alpha-actin expression. In chapter 4, we investigated the effect of CaMKII overexpression on the transcription of

skeletal alpha-actin (SKAA). We hypothesised that CaMKII overexpression would increase SKAA promoter activity and mRNA levels. The luciferase gene controlled by a SKAA promoter fragment was electro-transferred into *m. soleus* (SOL) and *m. gastrocnemius* (GM) of both legs. CaMKII was overexpressed in SOL and GM of the right leg only. In contrast to our hypothesis, luciferase activity measured in protein extracts of the CaMKII-transfected muscles SOL and GM was significantly lower compared to that in muscles transfected with the reporter only. SKAA mRNA level tended to be lower in SOL of the CaMKII transfected leg, but this was not significant. We concluded that CaMKII overexpression had a negative effect on SKAA gene transcription, but that the mechanisms underlying this effect require further investigation.

The results described in chapter 3 demonstrated that CaMKII overexpression *in vivo* decreased twitch contraction and relaxation time. However, it was unclear if direct CaMKII-dependent modification of the calcium release and re-uptake channels could have contributed to this effect. In chapter 5, we investigated whether CaMKII is activated more strongly when it's located in the vicinity of the RyR, and if increasing CaMKII concentration in sarcomeres is sufficient to explain decreased twitch contraction and relaxation times following CaMKII overexpression in rat skeletal muscle. A mathematical model of spatiotemporal sarcomeric [Ca^{2+}] dynamics coupled to a biochemical model of CaMKII activation was used. The model predicted substantial spatial gradients in CaMKII activity in sarcomeres of fast- and slow-twitch muscle fibres during single and repeated RyR openings. Increasing CaMKII concentration in the model to mimic CaMKII overexpression did not produce decreased twitch contraction and relaxation times as observed *in vivo* (chapter 3). It was suggested that this may be due to the fact that CaMKII is minimally activated during a twitch. As CaMKII overexpression was shown to increase SERCA2 expression in *m. soleus* muscle fibres (chapter 3), the effects of increased SERCA levels were also modelled. This resulted in a decreased relaxation time in the model. We concluded that a CaMKII overexpression-induced increase in SERCA expression, but not modification of SERCA, can at least partly explain the observed decrease in twitch contraction and relaxation times.

Considerations regarding overexpression of wild-type CaMKII by electro-assisted gene transfer

A few technical issues related to the approach used to overexpress CaMKII in rat skeletal muscle have not been explicitly addressed in the individual chapters, and will be discussed here.

The results of the transfection experiments described in this thesis demonstrate that CaMKII overexpression has effects on the expression of SERCA2 protein and skeletal alpha-actin promoter activity. This raises the question as to what the mechanisms are via which these effects were achieved. It is generally presumed that CaMKII affects cellular functions through its kinase activity. Because a wild-type CaMKII was overexpressed, the activation of the exogenous CaMKII likely depended on muscle recruitment-induced increases in calcium/calmodulin concentration. We attempted to determine whether exogenous CaMKII was activated after *in situ* stimulation of muscle, by comparing phospho^{Thr287} levels in stimulated and non-stimulated CaMKII-transfected muscles (chapter 3). Although increased phospho^{Thr287} levels of the exogenous β-CaMKII isoform was observed in some of these experiments, this was not consistent. It is also possible that Ca^{2+}/CaM-dependent, rather than Ca^{2+}/CaM-independent activity (which is reflected by phospho^{Thr287}-CaMKII) is increased in CaMKII overexpressing muscles. However, as Ca^{2+}/CaM-dependent activity cannot be measured, it cannot be concluded with certainty that any observed effects of CaMKII overexpression are due to increased (contraction-induced) kinase activity.

COXIV was measured as a marker of mitochondrial density, as its expression in skeletal muscle is increased by CaMKIV and PGC-1 overexpression (Wu et al., 2002, Jiang et al., 2010) , and therefore we hypothesised that COXIV expression would be increased in rat skeletal muscle by CaMKII overexpression. An increase in COXIV expression would not have demonstrated an increase in functional mitochondria, as these consist of many different proteins. However, if mitochondrial volume density had increased, COXIV protein levels would have been increased. As we observed no difference in COXIV protein level between control- and CaMKII-transfected muscles, we presume

that the volume of functional mitochondria was also not increased in CaMKII-transfected muscles.

Electroporation was used to induce uptake of plasmid DNA by the muscle fibres. This method is thought to result in the permeabilization of the cell membrane, allowing DNA molecules to enter the cytoplasm. Thus, the method depends on the induction of some degree of damage to the muscle fibres. To identify the effects of CaMKII overexpression, CaMKII-transfected fibres were compared to adjacent non-transfected fibres. As described in chapter 3, separation of these two fibre populations was based on visual inspection of CaMKII immunostaining intensity. Both fibre populations contained centrally-nucleated fibres, indicating ongoing regeneration. Therefore, it is conceivable that both populations have taken up the plasmid DNA, but that the 'non-transfected' fibres expressed the encoded CaMKII at much lower levels. This might be an explanation for the absence of a difference in COXIV expression. However, we did detect a significant difference in SERCA2 immunostaining intensity between transfected and non-transfected muscle fibres. Therefore, it seems likely that the difference in CaMKII expression levels achieved between 'CaMKII-transfected' and 'non-transfected' fibres is of a sufficient magnitude to expose CaMKII-dependent effects on protein expression.

Regulation of CaMKII activity in skeletal muscle

Although CaMKII is known to be activated during exercise in humans (Rose and Hargreaves, 2003), very little is known about the physiological regulation of this activity. In this section, we discuss the factors that regulate activation and deactivation of CaMKII in skeletal muscle.

CaMKII activation

A large degree of variation in levels of phospho^{Thr287}-CaMKII was observed in resting SOL and GM compared to the magnitude of the effect of *in situ* stimulation on the levels of phospho^{Thr287}-CaMKII in these muscles as reported in chapter 2. The relatively high variation in phospho^{Thr287} could be related to the fact that the muscles were not immediately sampled after the contraction protocols. However, this suggestion was not

supported by experiments described in chapter 3, whereby transfected muscles were freeze-clamped after two minutes of stimulation. In these experiments, no consistent increases in phospho^{Thr287} levels of the endogenous CaMKII isoforms were detected (Chapter 3, Fig. 2). Combined with the difference in resting levels of phospho^{Thr287}-CaMKII, this demonstrates that other factors than neural stimulation regulate CaMKII phosphorylation.

Passive stretch of mouse EDL and SOL *ex vivo* (i.e. without intact neural stretch reflex loops) increased phospho^{Thr287}-CaMKII in these muscles (Jensen et al., 2007), and may therefore be one such non-neural factor. However, as the muscles in the 'slack contraction' experiment (chapter 2) were not stretched and a substantial degree of variance in phospho^{Thr287}-CaMKII levels was still observed, this is unlikely to be one of the main factors causing the variance in phospho^{Thr287}-CaMKII levels in the muscles in our experiments. A possibility is that the calmodulin concentration differs in muscles from different animals. As reported in chapter 5, calmodulin concentration may be a limiting factor for CaMKII activation, and therefore a higher calmodulin concentration may lead to a higher CaMKII activity upon neural stimulation. Intramuscular calmodulin concentration doubled within two days of chronic electrostimulation of rabbit EDL (Antipenko et al., 1999), suggesting that calmodulin expression is in fact regulated by long-term muscle activity pattern. However, this would not explain the variation in the resting level of phospho^{Thr287}, as the animals used during our experiments were not trained and had similar activity patterns as they were kept in cages until the measurements were carried out. Therefore, it is still unclear what caused the variation in the phospho^{Thr287}-CaMKII levels in SOL and GM in our experiments.

The regulation of CaMKII activation may depend on the muscle phenotype. The modelling results of chapter 5 suggest that the higher amplitude of fast-twitch (FT) calcium transients activate CaMKII to a greater extend in FT muscle compared to slow-twitch (ST) muscle. This is supported by the experimental data reported in chapter 2, which demonstrated that CaMKII autophosphorylation was increased in fast-twitch GM, but not in slow-twitch SOL after *in situ* stimulation with 100 electrical pulses. To the best of our knowledge, no other study has investigated electrical stimulation-induced CaMKII phoshorylation in different muscle types. Further work is required to determine

whether the difference in stimulation-induced phospho^{Thr287}-CaMKII levels in FT and ST muscles persists during longer stimulation protocols reflecting exercise sessions.

In addition to affecting stimulation-induced CaMKII phosphorylation, muscle type also has an effect on basal levels of phospho^{Thr287}-CaMKII. Increased resting levels of phospho^{Thr287}-CaMKII levels were observed in skeletal muscle after a period of one-legged endurance training in humans (Rose et al., 2007b) and after chronic overloading of chicken muscle (Fluck et al., 2000b). As these training sessions increased the expression of genes involved in regulating oxidative metabolism and muscle size, respectively, these results suggest that the increased basal phospho^{Thr287}-CaMKII levels may be involved in establishing a new muscle phenotype. However, higher levels of basal phospho^{Thr287}-CaMKII were observed in rat low-oxidative white GM compared to high oxidative red GM (chapter 2) and in mouse fast-twitch, low-oxidative EDL muscle compared to slow-twitch high-oxidative SOL (Jensen et al., 2007). Therefore, basal phospho^{Thr287}-CaMKII levels in skeletal muscle is not simply higher as the oxidative capacity of the muscle increases, and different mechanisms may regulate resting CaMKII phosphorylation in trained and untrained muscle. It is currently unclear what the mechanisms are that lead to these increased basal levels of phospho^{Thr287}-CaMKII levels.

The development of autonomous CaMKII activity is dependent on the frequency of exposure of the enzyme to Ca^{2+}/CaM (De Koninck and Schulman, 1998). However, we observed no effect of neural activation frequency on phospho^{Thr287}-CaMKII in experiments where muscles at their active slack length were stimulated *in situ* (chapter 2). Furthermore, our simulations of CaMKII activity development suggested that the RyR opening frequency only has an effect on phospho^{Thr287}-CaMKII levels during the first few RyR openings (chapter 5). Therefore, the biochemical 'Ca^{2+}/CaM-frequency decoding' property of CaMKII appears to be relevant in whole muscle only during the initial RyR openings. This may be explained by the following three mechanisms: 1) the high frequency of calcium release in rat skeletal muscle compared to those used during the *in vitro* experiments by De Koninck & Schulman (13-250 Hz (Hennig and Lomo, 1985) versus 1-4 Hz (De Koninck and Schulman, 1998)), 2) the relatively slow deactivation of phosphorylated CaMKII after muscle stimulation (chapter 2) and 3) the

existence of a maximum level of autophosphorylated CaMKII in skeletal muscle (chapter 5).

One study has claimed that the frequency at which multiple contractions occur determines CaMKII activity. This conclusion was based on mathematical modelling and the observation that KN93, a pharmacological inhibitor of CaMK's, inhibited calcium release in single fast-twitch mouse muscle fibres when contractions occurred at 100 ms intervals, but not when contractions occurred at 5 second intervals (Aydin et al., 2007). However, CaMKII phosphorylation and activity were not measured during this study. Therefore, experiments that employ muscle contraction protocols with repeated contractions followed by measurement of CaMKII phosphorylation and/or activity are still required to confirm whether the frequency of muscle contraction has an effect on CaMKII activation.

It cannot be excluded that calcium influx from other sources than the SR is important for CaMKII activation. Extracellular calcium can enter the cytoplasm through store-operated calcium entry (via SR protein stromal interaction molecule 1 and the Orai1 calcium channel), excitation-coupled calcium entry and transient receptor potential channels (TRPC's) (Dirksen, 2009)). Although calcium influx through TRPC3 has been implicated in the activation of the calcineurin - nuclear factor of activated t-cells (NFAT) pathway (Rosenberg et al., 2004), very little is known about the magnitude of calcium influx through these channels and as of yet no implications for CaMKII activation can be derived.

The calcium concentration in myonuclei also increases during electrical stimulation of muscle fibres, but with slower dynamics compared to those in the cytoplasm (Liu et al., 2005). CaMKII-like activity has been demonstrated in nuclei isolated from chicken skeletal muscle (Fluck et al., 2000a). Some studies have claimed that CaMKII in the nucleus is phosphorylated after exercise based on immunohistochemical measures (Smith et al., 2008, Liu et al., 2005) but these data are not convincing as comparisons of staining intensities between different stained sections were made. Therefore, the results are likely to be affected by differences in staining quality and imaging settings between the different sections. It is currently unknown

which CaMKII isoforms are within the myonucleus, and therefore they cannot be distinguished on the western blots performed in chapters 2 and 3.

The roles of the different CaMKII isoforms and whether they are differentially localized in muscle fibres (as for example in cardiac myocytes (Ramirez et al., 1997)) requires further investigation. This knowledge will aid in determining which calcium micro-domains are important for their activation, and therefore help relate the physiological contraction-related stimuli to CaMKII-dependent downstream effects. Possibly, this could be done by overexpression of specific CaMKII isoforms, or targeting these isoforms for degradation using RNA interference (RNAi). These RNAi constructs would likely have to be targeted to the variable region within the association domain in order to knock-down individual isoforms (Hudmon and Schulman, 2002).

Oxidation of methionine residues 281/282 gives CaMKII autonomous activity in a mechanism parallel to autophosphorylation (Erickson et al., 2008). Like autophosphorylation of Thr287, oxidation of Met281/282 requires initial binding of Ca^{2+}/CaM and prevents blocking of the catalytic domain by the autoinhibitory domain (Erickson et al., 2008). Prevention of CaMKII oxidation by overexpression of methionine sulfoxide reductase A mitigated aldosterone-induced cardiac rupture in mice (He et al., 2011). This suggests that oxidation-dependent CaMKII activity has effects separate from those of autophosphorylation-dependent CaMKII activity in the heart. The role of oxidized CaMKII has not been the subject of any investigation in skeletal muscle. The formation of oxygen radicals is also induced in contracting skeletal muscle (Powers and Jackson, 2008), and it would therefore be of interest to determine whether oxidation of CaMKII occurs in skeletal muscle.

CaMKII de-activation

Much like the factors that regulate the activation of CaMKII, the factors that regulate the de-activation of CaMKII have received very little attention. Although Ca^{2+}/CaM is released from CaMKII when the intracellular [Ca^{2+}/CaM] is low, phosphorylation of CaMKII can only be reversed by the action of phosphatases. In both the 'two-minute isometric contractions' and 'slack contraction' experiments described in chapter 2, increased levels of phospho^{Thr287}-CaMKII in rat skeletal muscle were increased after

very brief stimulation (approximately 100 pulses) and remained up to 15 minutes after stimulation of rat skeletal muscle. This suggests that CaMKII autophosphorylation enables CaMKII to outlast the Ca^{2+}/CaM stimulus in skeletal muscle required for its initial activation.

One study observed increasing CaMKII activity and phospho^{Thr287}-CaMKII levels in rat fast-twitch EDL muscle *in situ* during stimulation up to 3 minutes after which both activity and phosphorylation decreased during continued stimulation (Rose et al., 2007a). In chapter 2, we reported that although phospho^{Thr287}-CaMKII levels for the δ_A-CaMKII isoform were increased after the activity required to determine optimum muscle length, phospho^{Thr287}-CaMKII was not increased for any of the CaMKII isoforms after a further two minute isometric contraction protocol (chapter 2). This suggests that muscle stimulation increases CaMKII phosphorylation, but also activates a pathway that dephosphorylates CaMKII, so that an elevated level of phospho^{Thr287}-CaMKII is only maintained after brief stimulation, and CaMKII is dephosphorylated during prolonged stimulation. The identity of this pathway for CaMKII dephosphorylation in skeletal muscle is currently unknown and requires further investigation.

CaMKII function in skeletal muscle

The current view in the literature is that CaMKII is involved in the regulation of mitochondrial biogenesis in skeletal muscle. However, our data indicate that basal levels of phospho^{Thr287}-CaMKII are higher in low-oxidative white compared to high-oxidative red rat *m. gastrocnemius medialis* (chapter 2). This result and the observation that CaMKII overexpression did not increase COXIV expression (chapter 3) indicate that increased CaMKII autophosphorylation and protein level are not sufficient to increase COXIV expression, which is essential to increase mitochondrial capacity. A possibility which has not yet been discussed is that CaMKII induced paracrine signalling that stimulated COXIV expression in non-transfected fibres as well as CaMKII-overexpressing fibres in the same muscle. In this case, there would not be a difference in COXIV staining intensity on the level of single fibres, but there would be a difference in COXIV expression between control- and CaMKII-transfected muscles detected by

western blot. However, we observed no difference on COXIV levels between control- and CaMKII-transfected muscles on a western blot (Fig. 1).

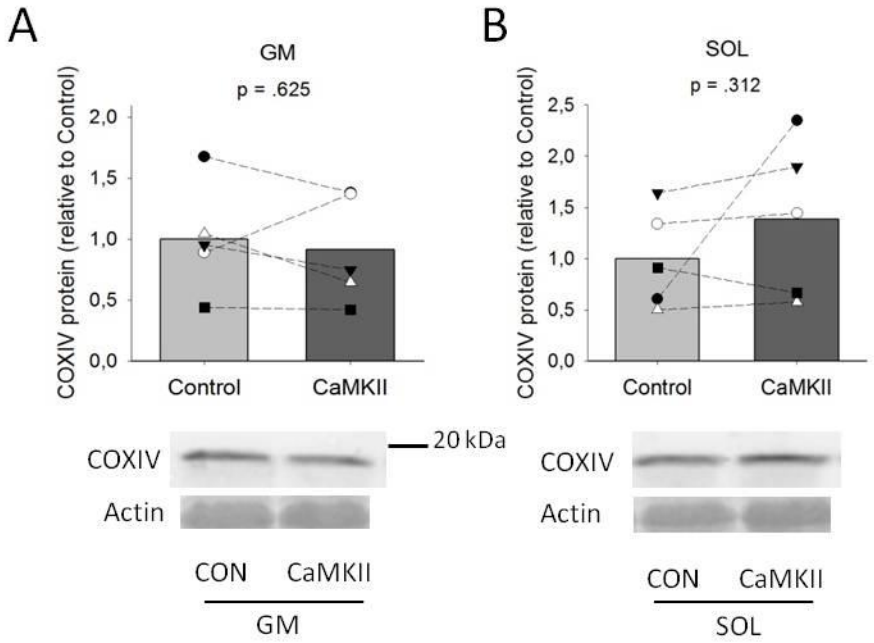

Figure 1: The effect of CaMKII overexpression on COXIV protein levels in whole muscle
Graphs display COXIV protein levels in control- (CON) and CaMKII-transfected GM (A) and SOL (B) as determined by western blotting followed by immunodetection. Bars represent mean COXIV and points represent the data obtained from individual samples. Points connected by a dashed line represent intra-animal muscle pairs. Significance of the difference between control- and CaMKII-transfected muscle s is indicated. Example blots are shown below the graphs. Actin was detected by ponceau s staining of the blot and serves as a loading control.

CaMKII per se may not be sufficient to induce mitochondrial biogenesis but it might be required in combination with other stimuli. This was indirectly suggested by the observation that the pharmacological CaMK inhibitor KN93 inhibited calcium-dependent activation of p38 mitogen-activated protein kinase (MAPK) in mouse epitrochlearis muscle *ex vivo* (Wright et al., 2007). MAPK inhibition had been demonstrated in the same study to inhibit calcium-dependent mitochondrial gene expression. However, this study did not measure the effect of CaMKII inhibition on mitochondrial expression directly. Inhibition of CaMKII in mouse skeletal muscle *in*

vivo has been carried out, but no data on mitochondrial gene expression have been reported (Murgia et al., 2009). It is therefore still unclear whether CaMKII is required for mitochondrial gene expression *in vivo*.

In contrast, the data presented in chapters 3 and 4 suggest that CaMKII overexpression is sufficient to increase SERCA2 protein expression and decrease skeletal alpha-actin promoter activity. This suggests that CaMKII does influence gene expression in skeletal muscle. Can putative CaMKII-dependent pathways of gene regulation explain increased SERCA2 expression? The SERCA2a promoter contains a serum response element (SRE) (Baker et al., 1998), and SRF overexpression resulted in decreased SERCA2 mRNA levels in cardiomyocytes (Zhang et al., 2001). This suggests that SRF might have an inhibitory effect on SERCA2 transcription. As CaMKII phosphorylates SRF at sites which either inhibit or stimulate its DNA binding (Fluck et al., 2000a, Rivera et al., 1993, Wheaton and Riabowol, 2004), our results suggest that CaMKII overexpression inhibited SRF DNA binding and decreased the activity of the SRF promoter construct (which contains three SREs (chapter 4)). Possibly by the same mechanism, CaMKII overexpression increased SERCA2 expression (chapter 3). In addition to SRF, MEF2 might be a CaMKII-regulated factor which mediates increases in SERCA expression (Lu et al., 2000a). A MEF2 site is present on the SERCA promoter, but SERCA promoter activity in cardiomyocytes is only increased when MEF2 is co-overexpressed with NFAT (Vlasblom et al., 2004), which is not known to be regulated by CaMKII. It should be noted that no increase in SERCA mRNA was observed in the CaMKII overexpression experiments in chapter 3. In conclusion, our data point to SRF as a possible mediator of CaMKII-dependent signalling in skeletal muscle *in vivo*, but its actual involvement still requires experimental verification.

Interestingly, δ-CaMKII expression increases in regenerating muscle (Abraham and Shaw, 2006). Increased calcium influx through damaged muscle membranes is thought to be an important factor in the pathophysiology of dystrophic muscle, as it may activate calcium-dependent proteases such as calpains (Whitehead et al., 2006). Stimulation of calcium removal from the cytoplasm by SERCA overexpression mitigates the dystrophic phenotype in skeletal muscle of mdx mice (Goonasekera et al., 2011). In the electroporated muscles in chapter 3, SERCA2 expression was decreased in the damaged

electroporated region compared to the non-electroporated region (Fig. 2). SERCA2 expression in *m.soleus* is also decreased during myotoxin-induced regeneration (Zador et al., 1996). In contrast, CaMKII overexpression increased SERCA2 protein expression (chapter 3, Fig. 6). Furthermore we observed a normalization of twitch contraction and relaxation time (chapter 3, Fig. 5). However, no improvements in maximal forceof the CaMKII-overexpressing muscles were observed seven days after transfection. Whether CaMKII overexpression decreases resting calcium level or calcium transients during muscle stimulation will require experimental verification. CaMKII could be co-overexpressed with a calcium-sensitive probe which allows for *in vivo* imaging (Rudolf et al., 2004). A comparison can then be made with the contra-lateral muscle overexpressing the probe only. To conclude, our results suggest that CaMKII may stimulate muscle regeneration by increasing SERCA2 expression.

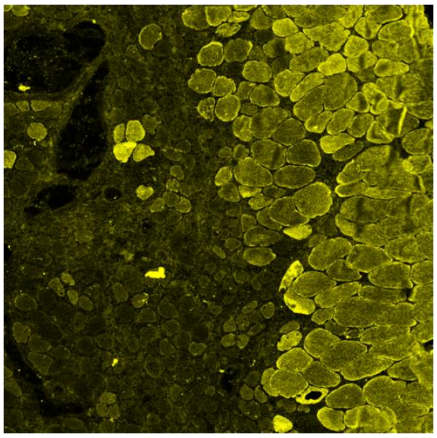

Figure 2: Decreased SERCA2 protein in damaged SOL fibres
Immunofluorescence image of SOL subjected to electroporation procedure and stained for SERCA2. The left half of the image contains small regenerating fibres, whereas the right half of the fibres is undamaged.The lower staining intensity in the regenerating fibres indicates lower SERCA2 levels in these fibres.

The combined results of the stimulation and transfection studies in this thesis suggest that CaMKII regulates gene expression in skeletal muscle, but that its muscle recruitment-dependent activity is limited to brief periods during and after muscle recruitment. The CaMKII activity pattern suggests that it is mostly involved in regulating acute processes during muscle contraction, rather than regulating processes such as the changes in gene expression during the recovery period after exercise. How can this apparent contradiction be explained? Firstly, it is not quite clear what the role of basal CaMKII activity in muscle is. As shown in chapter 2, there are differences in basal CaMKII phosphorylation between different muscle fibre types, and some studies have

demonstrated an increase in basal CaMKII phosphorylation after a period of training (Rose et al., 2007b, Fluck et al., 2000b). It may be that changes in basal activity and expression are related to adaptation (though not of oxidative capacity), and that contraction-induced activity is related to acute functions such as glycogen metabolism and calcium release. The latter hypothesis is supported by the modelling results described in chapter 5, which show that the increase in CaMKII activity is largest near substrates involved in regulating glycogen metabolism and calcium release (Singh et al., 2004, Leddy et al., 1993). Assessment of the impact of changes in basal CaMKII activity on muscle phenotype requires experiments in which skeletal muscles *in vivo* are overexpressing a constitutively active CaMKII-mutant. As of yet such experiments have not been carried out, but using the methods described in this thesis will provide relevant information regarding the role of CaMKII in skeletal muscle adaptation.

The role of calcium in mitochondrial gene expression

Calcium influx into skeletal muscle is generally considered to be a factor which stimulates mitochondrial biogenesis (Ojuka et al., 2003, Wright et al., 2007, Bruton et al., 2010). This raises the question as to what the mechanism is through which the calcium-dependent increases in mitochondrial biogenesis are achieved. Constitutively active calcineurin increases mitochondrial biogenesis in mouse skeletal muscle (Long et al., 2007, Jiang et al., 2010), and thus the effects of increasing calcium concentration may be mediated by calcineurin. However, an alternative explanation is that the effects of chronically elevated calcium concentration may be caused by indirect effects (i.e. not requiring calcium-dependent molecules). Increased cellular energy expenditure is likely required because calcium needs to be actively removed from the cytoplasm as continuously elevated calcium concentrations are associated with skeletal muscle pathology (Oberc and Engel, 1977). The resulting increase in intracellular AMP, and decrease in ATP concentration could activate AMPK, which appears to be sufficient to increase mitochondrial gene expression (Garcia-Roves et al., 2008). Alternatively, the increased calcium levels may have activated CaMK kinase (CaMKK), which activate AMPK in skeletal muscle (Jensen et al., 2007). CaMKK can be inhibited by KN93 (Jensen et al., 2007), and this might therefore explain the observed KN93-dependent

attenuation of mitochondrial gene expression in response to calcium (Wright et al., 2007).

A promising approach to test the physiological role of calcium in muscle adaptation without chronically elevating the intracellular calcium concentration is to use actomyosin-ATPase inhibitors to separate the calcium influx from muscle contraction (Dentel et al., 2005). This experiment cannot be carried out *in situ* or *in vivo*, as the actomyosin inhibitor would paralyse the diaphragm. Isolated muscles should be incubated with or without the inhibitor, and stimulated with a protocol that stimulates mitochondrial gene expression. A comparison with unstimulated muscles should identify whether physiologically relevant calcium transients can stimulate mitochondrial gene expression to the same extend as observed in contracting skeletal muscle.

Conclusion

The aim of this thesis was to elucidate the role of CaMKII in skeletal muscle adaptation *in vivo*. We conclude that CaMKII is activated by very brief stimulation in a recruitment frequency-independent manner, and that increased CaMKII protein levels increase SERCA2 expression, but not mitochondrial gene expression. We speculate that contraction-induced CaMKII activity may be mainly involved in regulating acute processes in skeletal muscle, and that chronic increases in CaMKII activity regulate long-term adaptation of skeletal muscle. The adaptations regulated by CaMKII are still unclear and deserve further investigation.